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Altering The Expression of Artemisinin Through Osmotic Manipulations

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Abstract— Artemisinin is an anti-malarial drug used in combination therapy to treat all malarial parasites in the blood stage. The expression of artemisinin within the plant *Artemisia annua* is only 1% of the dry weight. Methods for increasing the level of artemisinin within the plant were proposed. This paper looks into finding homologous enzymes across multiple species in order to find species where genetic manipulations will be useful. The second part of this paper looks at the use of osmotic stress to increase the reactive oxygen species in order to increase the amount of artemisinin within the plant. The database portion was successful in determining alternative species that genetic manipulation could be attempted on. The experimental portion was unable to shed light on the effects of a second reaction oxygen species on the production of artemisinin. However, the research did suggest that the level of artemisinin is increased during the recovery from stress.

Keywords—Artemisinin; *Artemisia annua*; Osmotic stress; Malaria; ACT

I. INTRODUCTION

The use of artemisinin to fight malaria is dependent on the plasmodium parasite not acquiring resistance to the drug. The acquisition of artemisinin resistance would be detrimental for the treatment of malaria. Unfortunately, artemisinin is in short supply, which increases the amount of unfinished drug cycles. Increasing the production of artemisinin within a plant will help decrease the chance that the parasite will acquire resistance to this treatment. This paper considers two ways of increasing the production of artemisinin either through genetic manipulation or external stress.

A. Malaria history

Malaria is caused by the plasmodium parasite, which is believed to be over 100,000 years old [1]. This parasite has constantly been evolving as human immune systems have evolved to fight them. One method of protecting against malaria is the condition of hemoglobin S (HbS). In its homozygous state HbS causes sickle cell anemia which can be deadly, however in its heterozygous state HbS protects against malaria [2]. The strain *Plasmodium falciparum* is attributed to 225 million cases of malaria every year and just shy of 1 million deaths per year [1].

There are five distinct species of the malarial parasite *Plasmodium*, that infects the human body. These include *falciparum*, *vivax*, *malariae*, *ovale*, and *knowlesi*. The method of infection for *P. falciparum* involves the human receiving a bite by a female mosquito. This releases a sporozoite into the skin. These sporozoites enter the capillaries and travel to the liver. From here the sporozoite can infect hepatocytes where they replicate during the next week. After a week they release merozoites. Once these merozoites enter the blood vessels, they invade red blood cells (RBCs), replicate, rupture the RBCs and this process repeats for 48 hours. Eventually the merozoites differentiate between male and female gametocytes, which fuse in the mosquito to form ookinetes. These ookinetes eventually differentiate into sporozoites and the process starts all over again [3]. While the parasite is in the liver most common antimalarial drugs cannot target the parasite, which leads to the patients relapsing after treatment [4]. This has led to many strains of malaria which are resistant to the common antimalarial agents. Artemisinin is a potential treatment for these strains of parasites, however ineffective use of artemisinin has led to strains resistant to artemisinin [3].

To curb the spread of malaria, individuals rely heavily on insecticides and insecticide treated bed nets. However, studies have been performed in order to determine the anopheles mosquito feeding preferences. Braack et. al showed that the anopheles mosquitos preferred to bite lower to the ground. They showed that protecting below the mid-calf significantly decreased the number of mosquito bites by up to 68%. This study also indicates that when laying on the ground, mosquitos would randomly bite people. However, they preferred to bite along the lower edge of the body. This research stresses the need to protect anyone sleeping close to the ground, as well as covering below the mid-calf when walking around at night. These techniques could help prevent the spread of malaria [5].

The CDC states the symptoms of malaria often resemble the symptoms of a common cold, or influenza. This makes diagnosis difficult in areas less prone to malaria. The “clinical” symptoms of malaria include three distinct stages. The cold stage usually consists of shivering and an overall cold feeling. The hot stage includes fever, headaches, and can even cause seizures. The sweating stage usually results in sweating, and tiredness. However, in most cases there is no

distinct division in the cycles and these symptoms can occur at any time of the attack. These attacks typically last between six and ten hours. The attacks happen multiple times, usually with one to two days between attacks. Furthermore, certain strains of malaria can lie dormant for a few years between attacks. [3]

Treatments for Malaria have evolved as the parasite has grown resistant to the treatments. The treatment for malaria used to be chloroquine, however, resistance to this started in the 1970s. The main treatment later became quinine. Artemisinin treatment has been shown to be more effective than quinine and is normally better tolerated than quinine. Furthermore, resistance to quinine is spreading in South-East Asia [6]. One of the current treatments of malaria is fixed-dose artemisinin combination therapy (ACT). ACT is used because of its effect against all five parasites in the blood stream. As a combination therapy the risk of parasites acquiring resistance is decreased [7].

B. *Artemisia annua*

A. annua is a species of plant commonly referred to as sweet wormwood. This plant is one of the few species that naturally produces artemisinin, furthermore, it is the only species where large-scale extraction of artemisinin has been deemed effective. *A. annua* has been used in many traditional Chinese remedies to treat fever, jaundice, headaches, vertigo, and nosebleeds [8]. The plant is native to many countries in Asia and eastern Europe, however, it has been naturalized all over the world. Artemisinin composes approximately 1% of the overall dry-weight of the leaves of *A. annua*. The typical plant normally grows between 1 and 2 meters tall.

C. Flooding stress

Kumar et. al. showed that flooding stress has been linked to a decreased amount of Cinnamate-4-hydroxylase (C4H) within the plant. The C4H is responsible for converting trans-cinnamic acid into p-coumaric acid. Trans-cinnamic acid is also a precursor to salicylic acid. The less C4H within the plant the more salicylic acid that can be produced. Their paper further showed that the amount of C4H increased due to drought stress, cold stress, and salinity stress [9]. Tariq Aftab et. al showed in their 2010 paper that increasing the salicylic content of the plant can increase the plant size by up to 20%. Furthermore, the photosynthetic rate of plants with increased salicylic acid was increased by 30%. The increased plant size would lead to an increased amount of artemisinin. More important than the plant size increase was the effects of salicylic acid on the enzymes in the artemisinin synthesis pathway [10]. Pu et. al. determined that an increased amount of salicylic acid leads to an increase in expression of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR). HMGR is part of the MVA pathway. Their work also showed that an increased amount of Salicylic acid increases the expression of Amorpho-4,11-diene synthase (ADS) [11]. ADS converts farnesyl diphosphate into amorpho-4,11-diene. This is a major branchpoint away from the other sesquiterpenes including: Squalene, 8-epi-cedrol, germacrene A, β -farnesene, and β caryophyllene. Salicylic acid was shown to flood the plant with reactive oxygen species which helps convert

dihydroartemisinin into artemisinin [11]. These findings all indicate that flooding stress should increase the amount of artemisinin within the plant.

D. Inspiration

The inspiration behind this research was two-fold. The overall goal of the research was to determine ways to increase the production of artemisinin within the plant *A. annua*. It has been shown that resistance to artemisinin combination therapy has begun appearing in small pockets [6]. Due to the low concentration of artemisinin within the plant, the extraction quantity is low per plant. This means that it is harder to obtain artemisinin based drugs for the treatment of malaria. ACT requires multiple doses, and if patients do not receive all doses due to shortages then the parasites have a chance to grow resistant to the treatment. If artemisinin was produced in higher quantities within the plant, then there would be less shortages. This paper looked into two ways of increasing artemisinin content.

1) Inspiration of the database

Gibson et. al. made a completely synthetic bacterium that was able to self-replicate and form colonies. They plan to use the results of their research to aid in the transplant of part to whole genomes [12]. This can be used to insert the coding DNA responsible for enzymes into the genomic DNA of the species. This would affectively allow a scientist to add all the enzymes required to a plant for the plant to make artemisinin. A database containing the homologous enzymes would help aid in the genetic manipulation of species to produce artemisinin.

2) Inspiration of the plant based experiment

The experiment drew its inspiration from the Kumar et al paper that indicated that flooding stress increased the amount of artemisinin. This process was determined to be caused by the increased amount of salicylic acid due to the decreased amount of C4H. Their research indicated that there was indeed an increase in plant size of the flooded plants. Their research also indicated that the leaf naturally expresses less C4H than the rest of the plant. As well as saplings produce less C4H than a mature plant. The top of the plant was shown to have more artemisinin than the lower leaves [9].

E. Hypothesis

Extensive research has shown that *A. annua* acclimates to stress, and over time the amount of artemisinin decreases. A method of reducing the plants ability to acclimate to the stress, and create a second round of reactive oxygen species should increase the amount of artemisinin within the plant. Cycling the flooding stress should help increase the amount of artemisinin over longer periods of time, as it would decrease the ability of the plant to acclimate to the stress. Furthermore, it is hypothesized that each time the plant's stress is stopped and restarted the increased reactive oxygen species should increase the amount of artemisinin within the plant. A database housing homologous enzymes in other species is believed to be helpful in determining species where genetic manipulation would be helpful.

II. MATERIALS AND METHODS

A. Homology database

As no database has a complete pathway for the production of Artemisinin as well as the effects of flooding stress on the plants, a database was formed in an attempt to cover this gap. This database cumulated multiple research papers, as well as MetaCyc in order to propose a pathway for the production of artemisinin in the plant *Artemisia annua*. When MetaCyc did not have the enzyme name, primary research papers were used in order to determine the enzyme's name. The database starts with trans cinnamic acid and its conversion to salicylic acid. The pathway from raw starting material to Artemisinin was also included in this database. Acetyl-CoA can travel down the MVA pathway into the Artemisinin pathway, or pyruvate and glucose-3-phosphate can travel down the MEP pathway into the Artemisinin pathway. Both the MVA and the MEP have the same final product, which is also the start point of the Artemisinin pathway. Once the pathway was determined Uniprot was used to determine the sequence of the enzymes. If Uniprot did not have the sequence, the sequence for the model organism *Arabidopsis thaliana* was used when available. The sequences were then BLAST analyzed to find similar enzymes. The list of enzymes was curated to remove results of the same species. Then the data was curated to remove results where the known catalytic activity differed from the catalytic activity in *Artemisia annua*. Finally, an identity score greater than 80 was chosen along with an E score less than 1×10^{-10} was chosen. This provided homologous enzymes that held mostly a conserved sequence.

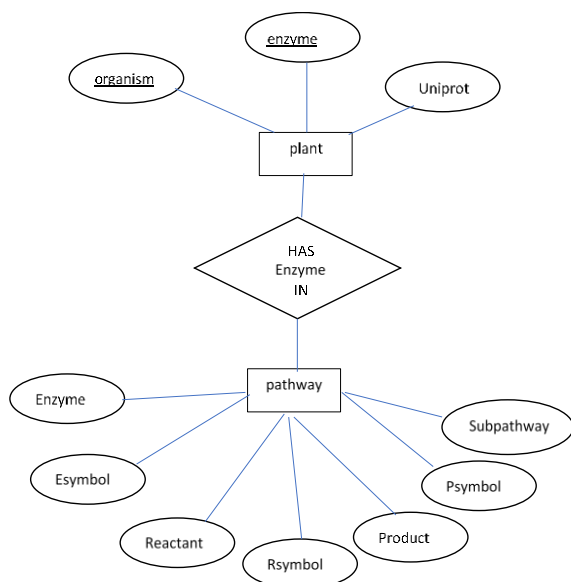


Figure 1: ER diagram of the database. This ERD represents the database. The two main tables in the database are pathway and plant. These tables are linked by their enzymes.

B. Relational database

Once this information was obtained, an SQL database was formed. This database had a table per enzyme. The name of each of these tables was the shortened name of the enzyme in *Artemisia annua*. These tables housed the species, the gene name in that species, the shortened gene name in that species, and the Uniprot ID. Once these tables were created, they were combined into the plant table. The plant table contains the organism name, the proposed enzyme synonym, and the Uniprot identification. Along with this information a table for each of the pathways was produced. These tables had the enzyme name, the reactant name, and the product name. Once the tables were created the data was inserted into the proper tables from the excel sheets that housed them. The resulting ER diagram can be seen in figure 1. Different statements were tested on the SQL to determine the limitations of the database.

C. Stressing the plants

Since flooding stress causes a release of reactive oxygen species (ROS), an experiment was proposed and performed in order to see the effects of cycling a flooding stress in order to release a second ROS burst throughout the plant. Nine plants were grown in a root riot cube for four weeks. At this point the plants were transferred into a four inch by four-inch plastic pot with 250 mL of Fertilome ultimate potting mix. The root riot cube was kept around the plant during and after transplant. All of the pots were punctured multiple times to allow the flow of water in and out of the pots. These nine plants were split into three groups and labeled. The first group was kept in tray one, which had extra dirt all around the pots in order to have spare dirt with the same soil water content as the soil in the pots. The second set of plants were placed in tray two which was full of water, which allowed water to sit above the soil in the pots. The third group was started in the second tray, and after 5 days was transferred into the first tray. It spent five days in the first tray before being transferred back into the second tray. The soil water content in the first tray was measured and maintained at $75\% \pm 5\%$. The soil water content requires the weight of dry mix to measure. To obtain the weight of the dry mix a sample of partially dry soil was placed into the oven at 90°C for three hours. The soil was returned to the beaker to be measured and weighed. The soil water content was determined by taking the current wet weight of the soil subtracting the weight of an equivalent amount of dry soil, and then dividing the total by the wet weight. This answer was then multiplied by one hundred to obtain a percentage. A soil water content of $75\% \pm 5\%$ was determined by Wang et al to be the normal soil water content [13]. Leaves were collected from each plant and dried in preparation for artemisinin extraction.

D. Extracting artemisinin

The dried leaves were weighed and then ground into a fine powder using a mortar and pestle. A tube was weighed, and the plant powder was added to the tube. The tube and the powder were weighed, and the weight of the powder was determined. 10 mg of hexane per 0.1 grams of leaf powder was used to extract the artemisinin. Once the hexane was

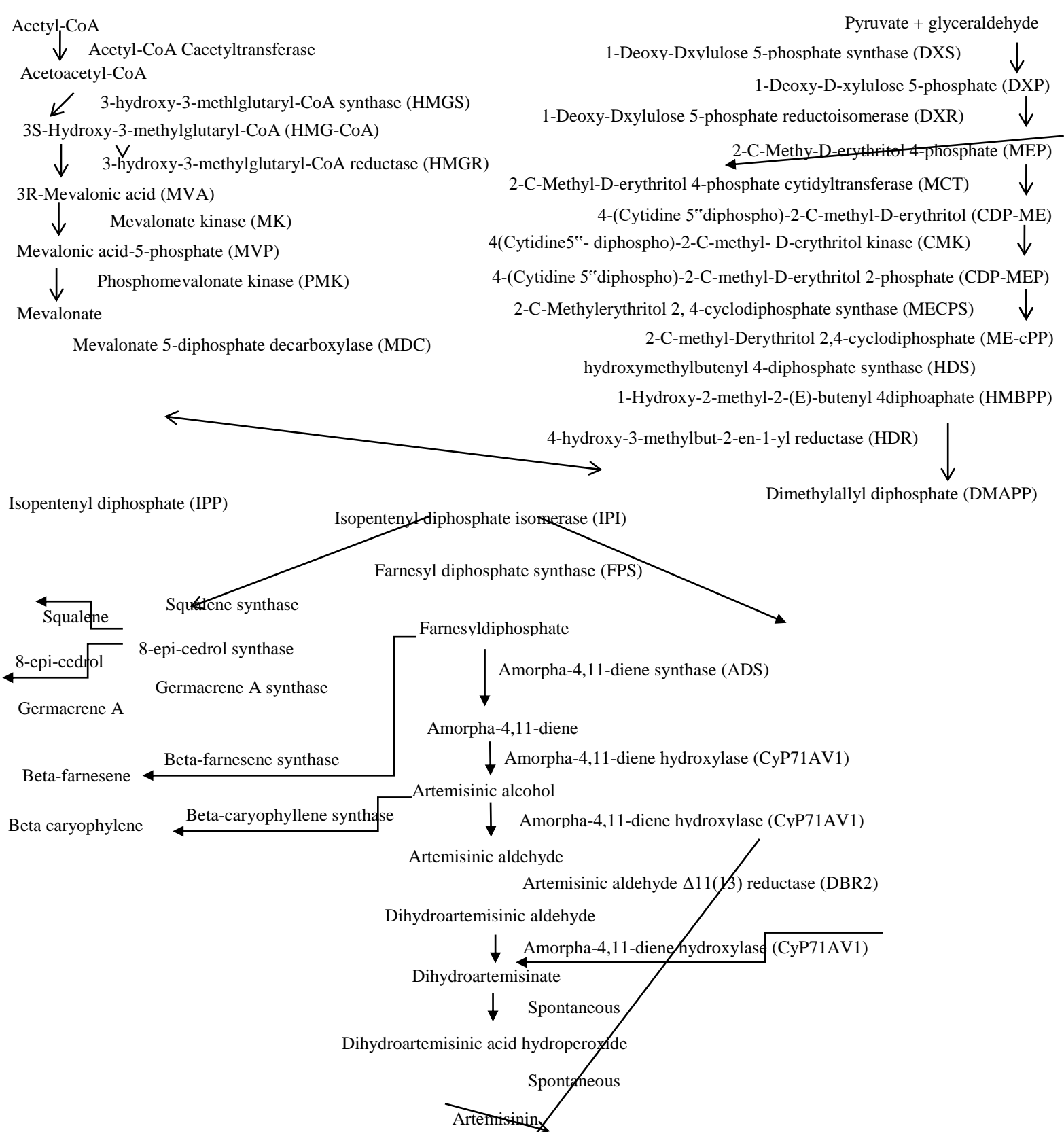


Figure 2: Proposed pathway for the formation of artemisinin. The proposed pathway for the formation of artemisinin. The top left is the MEP sub-pathway, the top right is the MVA sub-pathway. The pathways converge to form farnesyl diphosphate which follows the artemisinin pathway

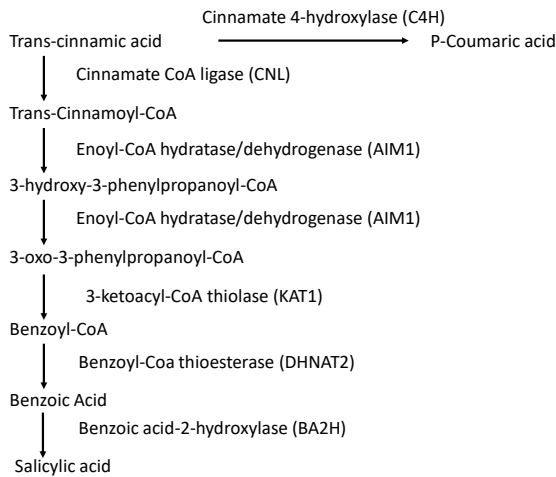


Figure 3: Trans-cinnamic acid to Salicylic acid

synthesis proposed pathway. The proposed pathway for the conversion of trans-cinnamic acid into salicylic acid. Some of the enzymes along the pathway have been proposed in primary papers.

added the tubes were heated at 50°C for 3 minutes. Afterwards, the tubes were kept in the fume hood overnight. The next day the leaf powder was removed, and the hexane was evaporated out at 50°C.

E. Determining the artemisinin content in the extract

Sreevidya and Narayana's method of using light spectrophotometry to determine artemisinin content was used as described in their 2008 paper. This paper calls for 1 mL of 5M HCL and 1 mL of 2% KI to be shaken until a yellow color forms. This was performed on an orbital shaker. Once a satisfactory yellow color was formed 0.5 mL of 0.01% solution of safranin O and 2 mL of 2 M NaOAc was added into the tubes. These tubes were then placed back on the orbital shaker for five minutes to insure maximum color change. Once this occurred the solution was diluted with 5.5 mL of deionized water. A standard was prepared by adding the 5 MHCL, 2%KI, safranin O and 2 M NaOAc to a cuvette. A spectrophotometer set at 521 nm was blanked using an empty cuvette. The absorbance of all solutions including the standard was subsequently determined. The artemisinin bleaches the solution due to the production of hydrogen peroxide. The artemisinin containing absorbance was subtracted from the standard and the difference was utilized in their equation [14]. Due to the high number of leaf extractions small samples were extracted. This forced the whole extracted leaf mass to be utilized in the determination of artemisinin.

F. Determining the root strenght

The quality of the root was qualitatively determined by applying a lifting force on the stem close to the soil. This was qualified by expressing the amount of resistance the roots provided in moving the plant. Further analysis of the length of root protruding from the bottom of the pot was performed as well.

III. RESULTS

A. Determination of the pathway

In order to determine the enzymes, a pathway had to be determined. This pathway successfully combined MetaCyc, NCBI, and primary research in order to form a plausible pathway. The complete pathway from raw starting material into artemisinin can be seen in figure 2. The pathway from trans-cinnamic acid to salicylic acid can be seen in figure 3. The pathway is split into four parts. The conversion of Cinnamate-4-hydroxylase to salicylic acid (figure 3), the MVA pathway (top right of figure 2), the MEP pathway (top left of figure 2), and the conversion of farnesyl diphosphate into artemisinin (bottom of figure 2). The MVA and MEP pathway are two ways that *A. annua* forms Dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate(IPP). These two chemicals are converted into farnesyl diphosphate by farnesyl diphosphate synthase (FPS). As long as the plant has one or the other pathway then the plant can produce both DMAPP and IPP. The pathway determined twenty-two enzymes. Across these twenty-two enzymes, 248 distinct species were determined. Of these twenty-two enzymes along the proposed path *A. annua* has been proven to have 14 of these enzymes the rest of the enzymes are still theorized to be in *A. annua*. The list of these enzymes can be seen in table 1. Unfortunately, the benzoate pathway is where most of the enzymes are still predicted, as this pathway is still not fully understood. For instance, the CNL enzyme has only been shown in two plant species, and the enzymes are dissimilar so only one of the enzymes made it into the database. Certain species have been removed from an enzyme list due to the low identity score. Further investigation into these species will be required before they are included into the database.

TABLE 1: Proven *A. annua* enzymes. This table shows the list of enzymes already discovered in *A. annua*, and that are involved in the biosynthesis of salicylic acid or artemisinin.

enzyme
ADS
CMK
CYP71AV1
DBR2
DXR
DXS1
FPS1
HDR
HDS
HMGR
HMGS
IPI
MCS
MCT

When looking at the three enzymes that take farnesyl diphosphate to artemisinin along with *A. annua* the database identified *A. absinthium* as another species with the required enzymes. However, the database was able to determine

multiple species with two of the three enzymes. These include *A. kurramensis*, *A. maritima*, and *Cynara cardunculus*. It also determined that as of right now we only know 19 plant species that have at least one of the required enzymes. Unfortunately, the model species *Arabidopsis thaliana* was not one of these species. Most of the homologous enzymes can be seen in the MVA and MEP pathway.

B. In Plant results

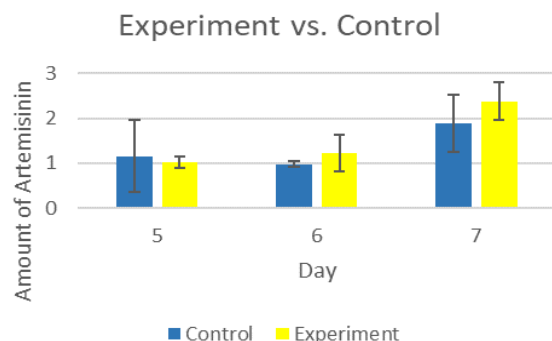


FIGURE 4: Experimental plant artemisinin vs. control. The results indicate that the amount of artemisinin is increased during recovery of the species.

The results show evidence that the amount of artemisinin is increased during the recovery from flooding stress. As seen in figure 4 the amount of artemisinin within the experimental group is less than in the control group while the experimental plant is undergoing flooding stress. After leaf samples were collected on day five, the plant was switched to the dry control condition. The amount of artemisinin increased between day 5 and 6 where the artemisinin was a little higher in the experiment group then the control group. By day seven the amount of artemisinin between day 5 and 7 in the experiment group had doubled, while the control group did not see a proportional increase. The amount of artemisinin in the experimental plant appeared to be higher than in the control plant.

C. Root strength

The health of the roots can determine the health of the plants. By day 12 the roots of the control plants were able to support the weight of the 4" pot including 250 mL of soil. On day 12 the flood group provided no resistance when lifted, however, some resistance was felt for the experimental plants. This step was repeated on day 21 and, as expected, the control roots were still able to lift the pot. Furthermore, these roots protruded from the holes on the bottom of the pot and measured 15 cm in length. By day 21 the flood group was able to lift the pot of soil. Surprisingly the pots had 11 cm in length roots protruding from the bottom. There were visually less roots protruding then the control group. Most plant species only grow roots as far as required in order to obtain water, so further exploration is required to

determine the cause of the root growth. It has been theorized as either a result of the salicylic acid or the demand for nutrients.



Figure 5: The change in pigmentation due to stress. This figure shows the leaves responding to the stress. The more stress the more yellow color to the leaves. Figure A is the control plant which shows the normal color of the leaves. Figure B is the flooded plant which shows yellowing especially in the back leaf. Figure C shows is the experimental plant which shows some yellowing, however it is not as yellow as the flooded plant.

D. Alterations in the color of the leaves

Viewing the leaves' color can aid in determining the amount of stress the plant is going through. Significant yellowing of

the leaves occurred across the plants in the flooding stress. As seen in figure 5 the plants in the flood stress were more yellow than the plants in the control stress. The yellowing indicates that the plants in the flood stress were experiencing stress. Furthermore, the experimental plants were more yellow than the control plants but less yellow than the flood plants. This is a result of the stress being stopped for a few days, and indicates that we were successful in cycling the stress.

IV. DISCUSSION

The database was successful in cumulating knowledge from MetaCyc, Uniprot, NCBI and primary papers in order to determine the pathway for the synthesis of artemisinin. The enzymes were used in Uniprot and BLAST in order to determine the homologous enzymes. This information was then placed into an SQL database. This database was successful in determining the species with homologous enzymes. The tables in the SQL database can be used alongside temporary tables to find information like which species has the required enzymes to convert farnesyl diphosphate into artemisinin. This database contains twenty-two different enzymes across four different pathways. It also identified 248 distinct plants with at least one homologous enzyme. The database was successful in discovering distinct species in which genetic manipulation may occur, specifically *A. absinthium* and *C. cardunculus*. However, further investigation is required before genetic manipulation is attempted. This includes the quantity of the enzymes produced, the sequence of Amorpha-4,11-diene hydroxylase (CYP71AV1), and determination if artemisinin is toxic to the rest of the plant. Komori et al. showed that CYP71AV1 has to have a serine at position 479 in order for the enzyme to complete all three of its enzymatic activities [8]. Further exploration into other enzymes requiring specific amino acids will also be necessary.

The in-plant portion was unable to shed light on the effectiveness of a second round of reactive oxygen species. Due to the successive number of days that leaves were pulled, low leaf samples were obtained each day. Since the extraction and quantification method were both based off of 0.1 grams of leaves and my samples were significantly less, the whole collected leaf mass was used. This added uncertainty since the mass size differed per leaf.

The plant portion did indicate, however, that artemisinin is produced during the recovery of the plant from flooding stress. However further studies will be required in order to obtain more significant data that would indicate this. A future experiment using greater extraction volumes and longer extraction times would help make the data more significant. Further research into the root growth is required in order to determine the cause of this growth. If further study determines that artemisinin is truly increased during the recovery of flooding stress, then these results can help further increase the artemisinin content at time of harvest. If seven days before harvest the plants are flooded, and the flooding stress is removed two days before harvest then the overall production of artemisinin would be increased.

V. CONCLUSION

This project was able to cumulate the entire known and proposed pathway for the production of artemisinin. The known and proposed enzymes were added to this pathway as well. This pathway was successful in determining homologous enzymes in other species where genetic manipulation could be used to produce artemisinin.

The plant based experiment should be modified in order to decrease the standard deviation of the numbers. This project illuminated changes that could help lower the standard deviation including: sister plants, larger collection sizes, and longer time between collection.

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